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Cytoskeletal perturbation leads to platelet dysfunction and thrombocytopenia in variant forms of Glanzmann thrombasthenia

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ABSTRACT

Several patients have been reported to have variant dominant forms of Glanzmann thrombasthenia, associated with macrothrombocytopenia and caused by gain-of-function mutations of *ITGB3* or *ITGA2B* leading to reduced surface expression and constitutive activation of integrin $\alpha_{IIb}\beta_3$. The mechanisms leading to a bleeding phenotype of these patients have never been addressed. The aim of this study was to unravel the mechanism by which ITGB3 mutations causing activation of $\alpha_{IIb}\beta_3$ lead to platelet dysfunction and macrothrombocytopenia. Using platelets from two patients carrying the β_3 del647-686 mutation and Chinese hamster ovary cells expressing different $\alpha_{IIb}\beta_3$-activating mutations, we showed that reduced surface expression of $\alpha_{IIb}\beta_3$ is due to receptor internalization. Moreover, we demonstrated that permanent triggering of $\alpha_{IIb}\beta_3$-mediated outside-in signaling causes an impairment of cytoskeletal reorganization arresting actin turnover at the stage of polymerization. The induction of actin polymerization by jasplakinolide, a natural toxin that promotes actin nucleation and prevents depolymerization of stress fibers, in control platelets produced an impairment of platelet function similar to that of patients with variant forms of dominant Glanzmann thrombasthenia. del647-686β_3-transduced murine megakaryocytes generated proplatelets with a reduced number of large tips and asymmetric barbell-proplatelets, suggesting that impaired cytoskeletal rearrangement is the cause of macrothrombocytopenia. These data show that impaired cytoskeletal remodeling caused by a constitutively activated $\alpha_{IIb}\beta_3$ is the main effector of platelet dysfunction and macrothrombocytopenia, and thus of bleeding, in variant forms of dominant Glanzmann thrombasthenia.

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Introduction

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), the main platelet receptor, is a heterodimeric calcium-dependent cell-surface glycoprotein expressed on platelets and megakaryocytes that plays a central role in platelet aggregation and thrombus formation.[1] Recent observations suggest that $\alpha_{IIb}\beta_3$ is also involved in proplatelet formation.[2,3] Integrin $\alpha_{IIb}\beta_3$ function depends on two different signal transduction pathways: inside-out and outside-in signaling. Under resting conditions, $\alpha_{IIb}\beta_3$ is expressed on platelets in a bent, inactive conformation. Upon platelet activation, inside-out signaling causes $\alpha_{IIb}\beta_3$ extension and headpiece opening, leading the receptor to assume an active conformation, to acquire the ability to bind its ligands, mainly fibrinogen, and to

initiate platelet aggregation.[4] On the other hand, ligand binding leads $\alpha_{IIb}\beta_3$ complexes to cluster, thus triggering outside-in signaling, with phosphorylation of the β_3 cytoplasmic tail, activation of signaling proteins (e.g. Src-family kinases and focal adhesion kinase), and ultimately reorganization of the actin cytoskeleton.[5] Cytoskeletal remodeling, with actin polymerization and depolymerization, is a finely regulated event,[6] and when altered it may lead to impaired platelet function and formation, as observed in patients with mutations of filamin A or with MYH9-RD.[7,8]

Mutations of *ITGA2B* and *ITGB3*, the genes coding for integrins α_{IIb} and β_3, generate Glanzmann thrombasthenia (GT), an autosomal recessive bleeding disorder characterized by absent platelet aggregation and a normal platelet count and volume, due to quantitative or qualitative defects of $\alpha_{IIb}\beta_3$. Heterozygous carriers of GT are usually asymptomatic because 50% of normal $\alpha_{IIb}\beta_3$ is sufficient for platelet aggregation,[9] but rare autosomal dominant variants of GT, with platelet dysfunction and macrothrombocytopenia, have been associated with gain-of-function mutations of *ITGA2B* or *ITGB3* leading to reduced expression and constitutive activation of $\alpha_{IIb}\beta_3$.[3,10]

We have previously described a hereditary Glanzmann-like platelet disorder transmitted in an autosomal dominant way, associated with macrothrombocytopenia and a bleeding diathesis, due to a heterozygous G>C transversion (c.2134+1G>C) of *ITGB3* leading to the deletion of exon 13 and to the loss of 40 amino acids (del647-686) of integrin β_3.[11] This mutation was the first-described, naturally-occurring deletion of the β-tail domain (β-TD) of integrin β_3, the membrane-proximal portion of the extracellular domain of the protein. β-TD contributes to maintain the bent, inactive conformation of $\alpha_{IIb}\beta_3$;[12] in fact, the loss of its disulfide bonds causes constitutive activation of $\alpha_{IIb}\beta_3$,[13-15] but no information is available on its role in outside-in signaling. Recently, a Japanese family carrying a heterozygous *ITGB3* c.2134+1G>A transversion leading to the same integrin β_3 deletion and a similar phenotype was reported.[16]

We have previously reported that del647-686 integrin β_3 leads to constitutive activation of $\alpha_{IIb}\beta_3$ in patients' megakaryocytes,[3] a finding recently confirmed in transfected 293T cells.[16]

A few other heterozygous patients with gain-of-function mutations of *ITGB3*, mucocutaneous bleeding and macrothrombocytopenia have been reported[17-19] suggesting that, independently of the mutation, constitutive activation of $\alpha_{IIb}\beta_3$ leads to platelet dysfunction and macrothrombocytopenia by a common mechanism that, however, has never been addressed.

Here we show that constitutive activation of integrin $\alpha_{IIb}\beta_3$ decreases surface expression of the complex through receptor internalization and permanently triggers outside-in signaling, which leads to altered cytoskeletal reorganization that is the main effector of platelet dysfunction and macrothrombocytopenia, and thus of bleeding, in autosomal dominant GT variant forms.

Methods

Blood samples were taken from healthy volunteers and from two patients carrying the *ITGB3*c.2134+1G>C mutation.[11] All subjects gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the ethical committee of the University of Perugia.

Construction of the expression vectors, mutagenesis and transfection

Expression vectors were obtained[20] and Chinese hamster ovary (CHO) cells were transfected as described in the *Online Supplementary Data*. All experiments were performed 2 days after transfection.

Surface biotinylation, β_3 immunoprecipitation and western blotting

Surface proteins on CHO cells and platelets were biotinylated, and integrin β_3 was immunoprecipitated and analyzed by western blotting as described in the *Online Supplementary Data.*

$\alpha_{IIb}\beta_3$ expression and internalization, and $\alpha_v\beta_3$ expression

$\alpha_{IIb}\beta_3$ expression on resting or activated platelets or CHO cells[21] was assessed by flow cytometry[22] or by western blotting after biotinylation of membrane proteins and β_3 immunoprecipitation. $\alpha_v\beta_3$ expression was assessed by flow cytometry.[22] Internalization of $\alpha_{IIb}\beta_3$ was assessed by flow cytometry as described elsewhere.[23] Platelet fibrinogen content was quantified by enzyme-linked immunosorbent assay (GenWay Biotech. San Diego, CA, USA). Details are given in the *Online Supplementary Data*.

Adhesion assay

CHO cells and platelets were layered on human fibrinogen. Platelets were also layered on human von Willebrand factor after treatment with 0.1 U/mL human α-thrombin.[18,24] Details are given in the *Online Supplementary Data*.

Protein phosphorylation

CHO cells and platelets were plated on human fibrinogen and protein phosphorylation was assessed as described in the *Online Supplementary Data*.

Clot retraction

Clot retraction was assessed with CHO cells and platelet-rich plasma[25] as described in the *Online Supplementary Data*.

Actin polymerization

Actin polymerization in CHO cells and platelets was assessed by flow cytometry as described in the *Online Supplementary Data*.

Analysis of cytoskeletal proteins

The cytoskeleton was extracted from either resting platelets or platelets that had been stimulated with thrombin as described elsewhere[26]. Cytoskeletal proteins were separated on an acrylamide-gel and stained with Comassie-blue[26]. For details see the *Online Supplementary Data*.

Mass spectrometry

Protein digestion and peptide analysis were performed as reported previously.[27] Databases were searched using MASCOT v.2.2 and MyriMatch v.2.1.87.[28] Protein assembly for MyriMatch analysis was made using IDPicker v.3.0.[29] Spectral-counts were used for semiquantitative comparisons between controls and patients. For details see the *Online Supplementary Data*.

Effect of the perturbation of actin polymerization on platelet function

$\alpha_{IIb}\beta_3$ activation, platelet aggregation,[30] clot retraction and spreading on fibrinogen[30] were assessed in platelets treated with

jasplakinolide to induce actin polymerization[31] as described in the *Online Supplementary Data*.

Construction of retroviral vectors, retrovirus production and megakaryocyte infection

The bicistronic pMYs-IRES-GFP/α_{IIb}, pRetroX-IRES-DsRedExpress/β_3 and pRetroX-IRES-DsRedExpress/$\beta_3$del647-686 expression vectors were obtained, retroviruses produced and megakaryocytes double-infected[32] as described in the *Online Supplementary Data*.

Megakaryocyte spreading, proplatelet formation and morphology

Spreading and proplatelet formation in murine megakaryocytes[32] were evaluated by immunofluorescence.[3] Proplatelet morphology in human blood was analyzed by immunofluorescence as described elsewhere.[3,33] For details see the *Online Supplementary Data*.

Effect of the perturbation of actin polymerization on proplatelet formation

Human megakaryocyte cultures[3] were treated with jasplakinolide (1 μM) for 10 min and then cultured for 16 h. Proplatelet formation, spreading on fibrinogen, proplatelet tip number and diameter were assessed as described above.

Structural consequences of del647-686

To unravel the possible structural consequences of del647-686 the three-dimensional structure of the β-TD was derived from the $\alpha_{IIb}\beta_3$ structure (PDBID-code 4G1E) and visualized using PyMOL (DeLano Scientific, San Carlos, CA, USA).[34]

Statistical analysis

Data are expressed as means ± standard deviation. An unpaired t-test or the two-way ANOVA with the Bonferroni post-test was applied, where appropriate, using GraphPad Prism version 5.00 (GraphPad Software,

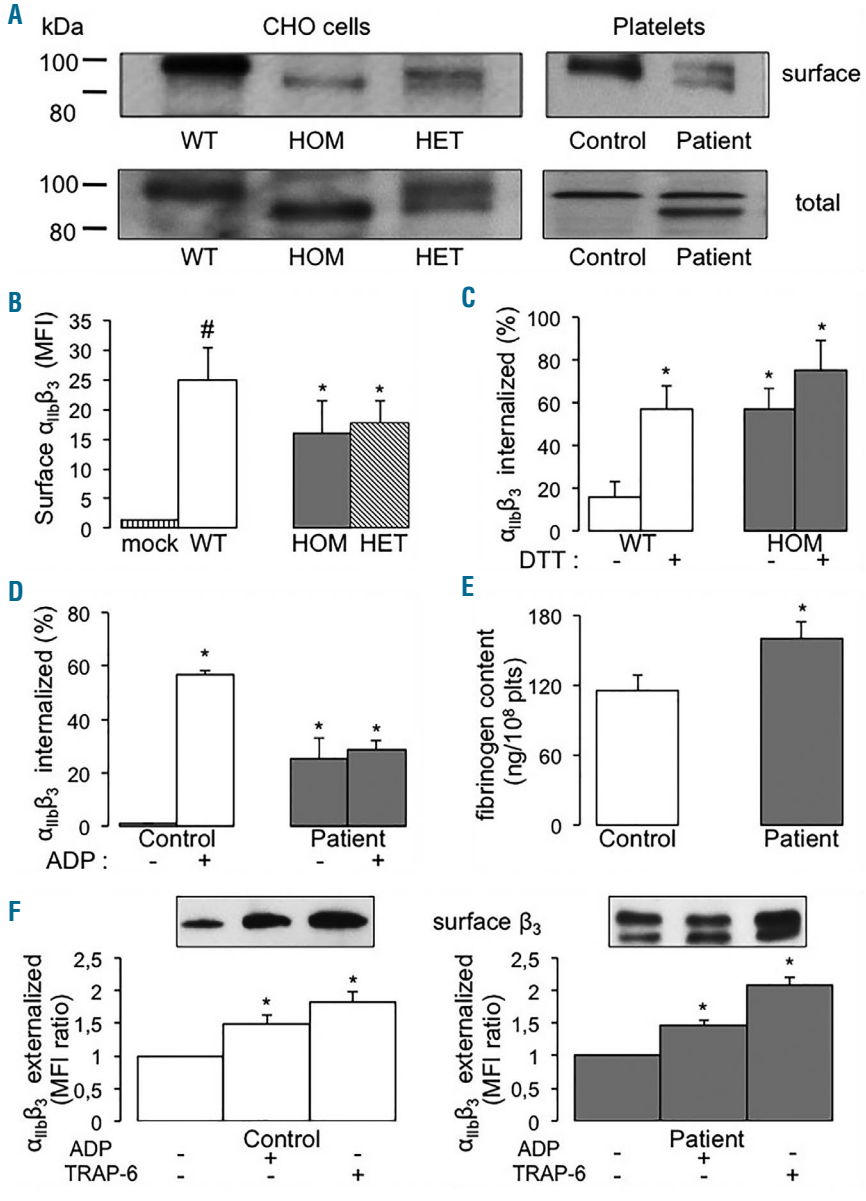

Figure 1. $\alpha_{IIb}\beta_3$ surface expression. (A) Western blotting of biotinylated (surface) and not-biotinylated (total) integrin β_3 of CHO cells expressing wild-type β_3 (WT), del647-686 β_3 (HOM) or both (HET) and of control and heterozygous patients' platelets. Biotinylated proteins were identified using HRP-conjugated avidin. The first band is wild-type β_3 and the second is mutant β_3. Relative quantification (arbitrary units), CHO cells: WT=1, HOM=0.18±0.02, HET=0.24±0.09 (n=5, *P*<0.05 *vs.* WT); Platelets: control=1, Patient=0.60±0.01 (n=5, *P*<0.05 *vs.* control). Total integrin β_3 expression was measured in not-biotinylated CHO cell and platelet lysates, probing membranes with a mouse anti human-β_3 monoclonal antibody. The total amount of integrin β_3 was comparable. Relative quantification (arbitrary units) CHO cells: WT=1, HOM=0.89±0.06, HET=0.97±0.1 (n=5, *P*=ns *vs.* WT); Platelets: Control=1, Patients=1.2±0.2 (n=5, *P*=ns *vs.* control). (B) $\alpha_{IIb}\beta_3$ mean fluorescence intensity (MFI) in CHO cells transfected with empty vectors (mock) or expressing wild type (WT), del647-686 (HOM) and both (HET) integrin β_3 (**P*<0.01 *vs.* WT, *#P*<0.01 *vs.* mock). $\alpha_{IIb}\beta_3$ MFI in CHO cells is comparable with $\alpha_{IIb}\beta_3$ MFI in platelets (22.1±3.2 in control platelets and 11.9±4.1 in patients' platelets). (C) Internalized $\alpha_{IIb}\beta_3$ in wild type and mutant CHO cells, activated or not with DTT (**P*<0.05 *vs.* resting WT). (D) Internalized $\alpha_{IIb}\beta_3$ in control and patients' platelets, stimulated or not with ADP (**P*<0.05 *vs.* resting control). The same experiments were conducted using TRAP-6 obtaining comparable results (*data not shown*). (E) Fibrinogen content in patients' platelets compared with controls as measured by enzyme-liked immunosorbent assay (**P*<0.05 *vs.* control). (F) Western blotting of biotinylated β_3 and flow cytometry of $\alpha_{IIb}\beta_3$ in control and patients' platelets, resting or stimulated with ADP or TRAP-6. Biotinylated proteins were identified using HRP-conjugated avidin.

San Diego, CA, USA). Differences were considered statistically significant when $P<0.05$.

Results

β_3 mutations and $\alpha_{IIb}\beta_3$ activation

CHO cells transfected with normal α_{IIb} and with four different mutant β_3 subunits express a constitutively activated $\alpha_{IIb}\beta_3$ receptor on their surface, i.e. a receptor able to bind PAC-1 and fibrinogen under resting conditions (*Online Supplementary Figure S1A,B*), confirming previous findings.[3,17-19]

Gain-of-function mutant β_3 reduces surface expression of $\alpha_{IIb}\beta_3$ by enhancing its internalization

Western blotting and flow-cytometry showed decreased surface expression of β_3 but a normal amount of β_3 in cell lysates, both in CHO cells expressing del647-686 integrin β_3 and in heterozygous patients' platelets (Figures 1A,B). Confocal microscopy of integrin β_3-transfected CHO cells showed that mutant β_3 is localized predominantly in the cytoplasm, while normal β_3 is localized predominantly on the cell surface (*Online Supplementary Figure S2*). Mutant $\alpha_{IIb}\beta_3$ co-localized with concavalin A and with WGA as shown by fluorescence microscopy, confirming normal synthesis and maturation (*Online Supplementary Figure S3*). Moreover, in patients' platelets, cytoplasmic $\alpha_{IIb}\beta_3$ co-localized with P-selectin in α-granules but also localized in other cytoplasmic structures, probably the open canalicular and dense tubular systems[35] (*Online Supplementary Figure S4*).

The fraction of internalized $\alpha_{IIb}\beta_3$ was significantly high-

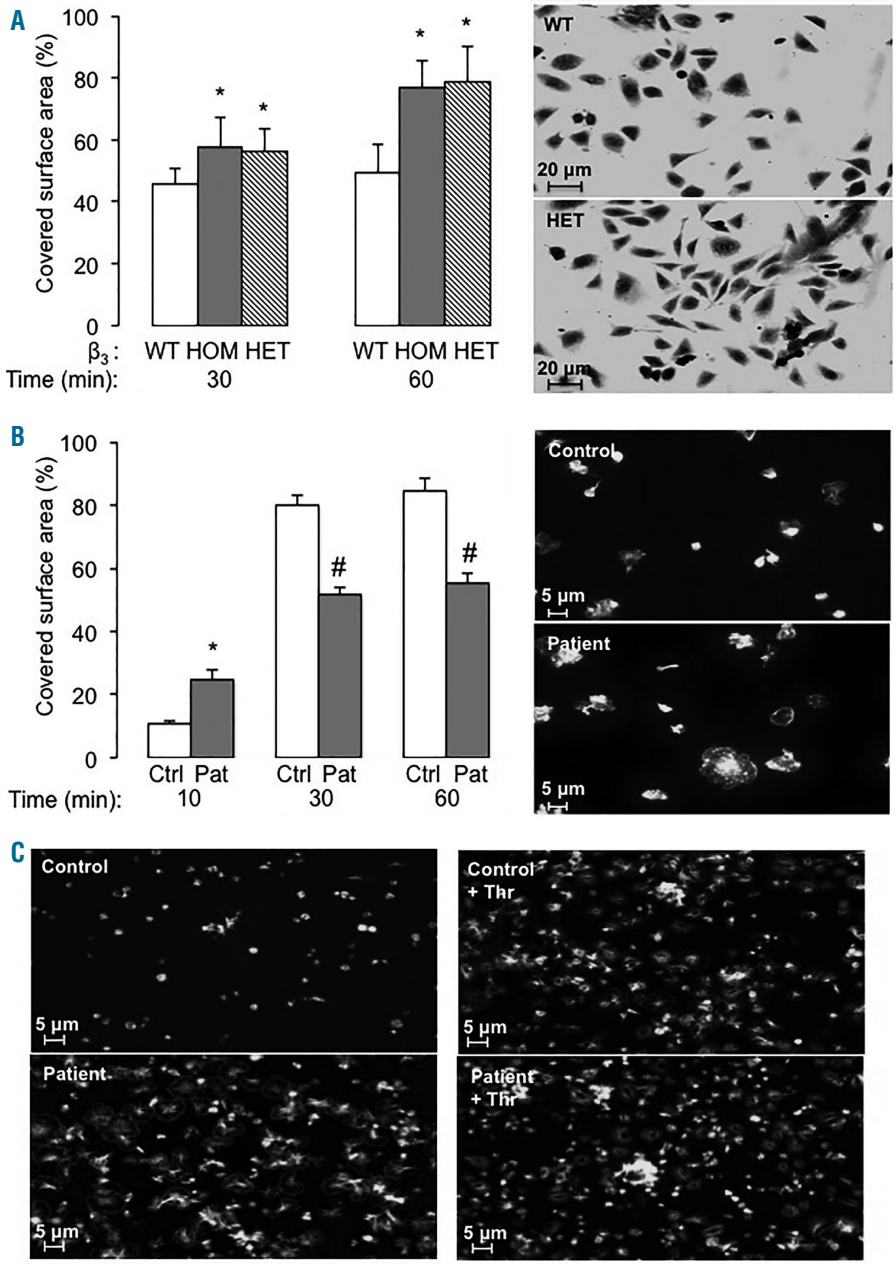

Figure 2. Spreading. (A) **Spreading of wild type (WT), mutant homozygous (HOM) and heterozygous (HET) integrin β_3-expressing CHO cells after 30 and 60 min of adhesion to fibrinogen (n=3, *$P<0.05$ *vs.* WT).** Representative images of WT and HET integrin β_3-expressing CHO cells after 60 min of adhesion to fibrinogen. The total surface covered was measured in 20 different high magnification microscopic fields. Mock-transfected CHO cells adhered to fibrinogen but did not spread. CHO cell adhesion on fibrinogen was comparable using the different cell lines: WT 30 min: 53.4±6.8 n. of adherent cells; WT 60 min: 43.5±5.4 n. of adherent cells; HOM 30 min: 52.5±7.9 n. of adherent cells; HOM 60 min: 51.7±5.3 n. of adherent cells; HET 30 min: 47.6±5.4 n. of adherent cells; HET 60 min: 51.6±8.4 n. of adherent cells. (B) Spreading of control (Ctrl) and patients' (Pat) platelets after 10, 30 and 60 min of adhesion to fibrinogen (n=5, *$P<0.05$, #$P<0.01$ *vs.* control). Representative images of control and one patient's platelets after 10 min of deposition on fibrinogen. Control and patient's platelet adhesion on fibrinogen was comparable: control 10 min: 9.5±2.0 n. of adherent platelets; control 30 min: 51.3±11.4 n. of adherent platelets; control 60 min: 69.9±7.2 n. of adherent platelets; Patient's 10 min: 11.5±3.1 n. of adherent platelets; Patient's 30 min: 48.7±5.3 n. of adherent platelets; Patient's 60 min: 59.6±8.7 n. of adherent platelets. (C) Representative images of control and patient's platelets after 30 min of deposition on von Willebrand factor under resting conditions or stimulated with human α-thrombin (+Thr). Both adhesion and spreading of patient's platelets under resting conditions were increased with respect to control: control: 52.5±6.2 n. of adherent platelets; 5.2±2.6% of spreading (% of covered surface); Patient's: 116.0±9.8 n. of adherent platelets; 55.2±10.6% of spreading (% of covered surface). After stimulation with thrombin both adhesion and spreading of patient's platelets were comparable with those of control platelets: control Thr: 198.1±11.0 n. of adherent platelets; 73.5±12.4% of spreading (% of covered surface); Patient's Thr: 214.8±12.9 n. of adherent platelets; 69.7±15.6 % of spreading (% of covered surface). Platelets were stained with FITC-conjugated phalloidin. Every microscopic field covers 0.14 mm^2.

er in unstimulated CHO cells expressing mutant β_3 than in cells expressing wild-type β_3 (Figure 1C). Similar findings were observed with resting patients' platelets as compared with control platelets (Figure 1D). In accordance, patients' platelets contained more fibrinogen than control platelets (Figure 1E) and mutant $\alpha_{IIb}\beta_3$-expressing CHO cells internalized fibrinogen under resting conditions differently from CHO cells expressing normal $\alpha_{IIb}\beta_3$ that required activation with DTT to internalize it (*Online Supplementary Figure S1C*). However, upon platelet stimulation with ADP or TRAP-6, the internal pool of $\alpha_{IIb}\beta_3$ of patient's platelets externalized regularly showing that it is correctly recycled after internalization (Figure 1F).

$\alpha_v\beta_3$ expression on patient's platelets was comparable to that on control platelets (control 34.9±8.0% *versus* patients 37.6±7.1%, *P*=ns).

Constitutively activated $\alpha_{IIb}\beta_3$ triggers outside-in signaling

CHO cells expressing mutant β_3 spread faster on fibrinogen than wild-type cells (Figure 2A). Spreading of patients' platelets was initially faster (Figure 2B), but after 30 min became defective[11]. Moreover, patients' platelets spread spontaneously on von Willebrand factor, unlike control platelets that required $\alpha_{IIb}\beta_3$ activation to undergo full spreading[18,24] (Figure 2C).

Integrin β_3 Tyr773 and Tyr785, as well as focal adhesion kinase, were constitutively phosphorylated in mutant CHO cells, while they were phosphorylated only after spreading on fibrinogen in control cells. Similarly, constitutive β_3 and focal adhesion kinase phosphorylation was observed in resting patients' platelets but not in control platelets (Figure 3A-C).

Finally, clot retraction induced by CHO cells expressing the mutant receptor was decreased (Figure 3D) as well as clot retraction of patients' PRP (Figure 3E).

Constitutive $\alpha_{IIb}\beta_3$ activation leads to impaired cytoskeletal reorganization

The content of polymerized actin (F-actin) of CHO cells expressing each of the heterozygous, β_3-activating mutants described so far[11,17-19] together with normal α_{IIb} was higher than that of CHO cells expressing normal $\alpha_{IIb}\beta_3$, and it did not increase after stimulation with DTT (Figure 4A). Similarly, F-actin content was significantly higher in resting patients' platelets as compared with con-

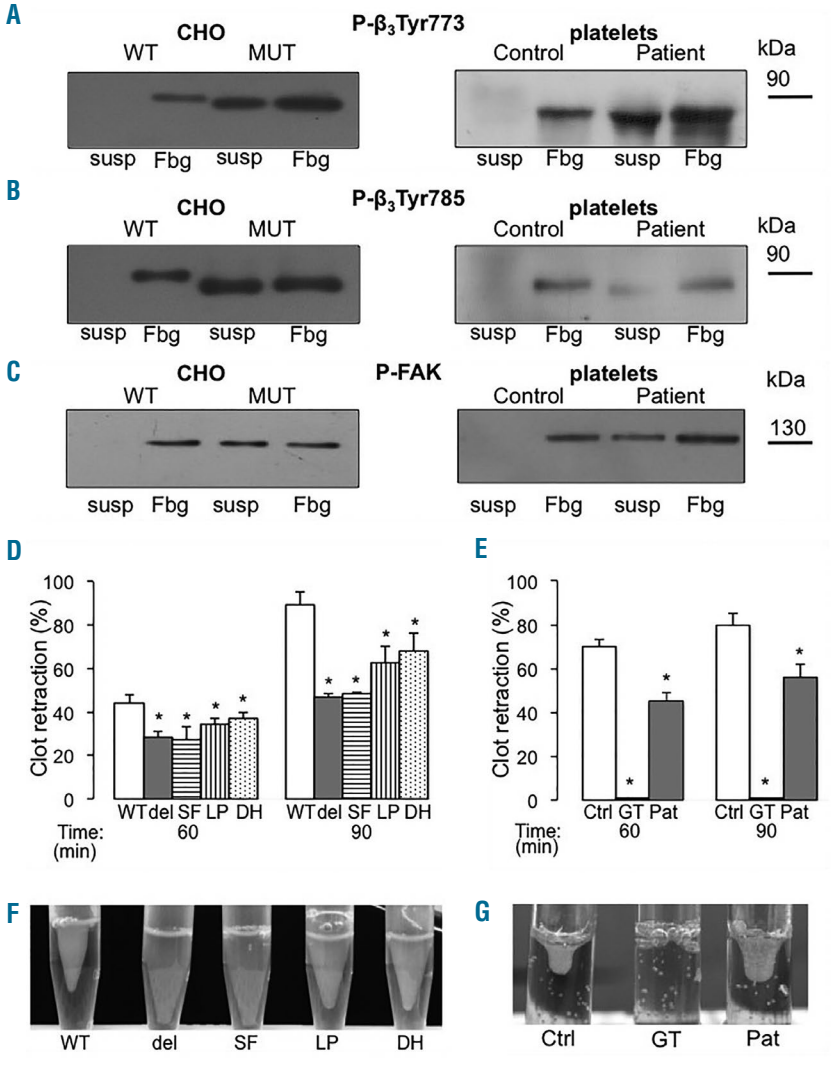

Figure 3. $\alpha_{IIb}\beta_3$-triggered outside-in signaling. (A) β_3 Tyr773 phosphorylation of wild-type (WT) and mutant (MUT) integrin β_3-expressing CHO cells and of control and one patient's platelets in suspension (susp) and after adhesion to fibrinogen (Fbg). Relative quantification (arbitrary units), CHO cells: WT susp=1, WT Fbg=29.6±4.7, MUT susp=55.3±6.8, MUT Fbg,=69.6±12.3 (n=5, *P*<0.05 *vs.* WT); Platelets: control susp=1, control Fbg=5.1±1.5, patient susp=7.1±1.8, patient Fbg=8.6±1.3 (n=5, *P*<0.05 *vs.* control). (B) β_3 Tyr785 phosphorylation of wild-type and mutant integrin β_3-expressing CHO cells and of control and one patient's platelets in suspension and after adhesion to fibrinogen. Relative quantification (arbitrary units), CHO cells: WT susp=1, WT Fbg=41.3±2, MUT susp=62.3±7.5, MUT Fbg=65.7±5.5 (n=5, *P*<0.05 *vs.* WT); Platelets: control susp=1, control Fbg=5.7±1.6, patient susp=2.4±1.1, patient Fbg= 2.9±0.6 (n=5, *P*<0.05 *vs.* control). (C) Focal adhesion kinase (FAK) phosphorylation of wild-type and mutant integrin β_3-expressing CHO cells and of control and one patient's platelets in suspension and after adhesion to fibrinogen. Relative quantification (arbitrary units), CHO cells: WT susp=1, WT Fbg=7.9±2.5, MUT susp=5.2±0.7, MUT Fbg=5.1±0.3 (n=5, *P*<0.05 *vs.* WT); Platelets: control susp=1, control Fbg=3.9±0.8, patients susp=2.1±0.7, patients Fbg=4.5±0.9 (n=5, *P*<0.05 *vs.* control). (D) Clot retraction induced by wild-type (WT) and the different mutant integrin β_3-expressing CHO cells after 60 and 90 min of incubation at 37°C (**P*<0.01 *vs.* WT). (E) Clot retraction induced by control platelets (Ctrl), Glanzmann thrombasthenia platelets (GT) and patients' (Pat) platelets after 60 and 90 min of incubation at 37°C (*p<0.01 *vs.* control). (F) Representative images of WT and the different mutant integrin β_3-expressing CHO cells after 90 min of incubation at 37°C. (G) Representative images of platelets from a control, a subject with Glanzmann thrombasthenia and one patient after 90 min of incubation at 37°C.

trol platelets. Consistently, stimulation with ADP did not significantly increase F-actin content of patients' platelets while it doubled it in control platelets (Figure 4B).

Cytoskeletal protein content was higher in resting patients' platelets than in resting control platelets. Moreover, electrophoresis showed three overexpressed bands in patients' platelets when compared to resting control platelets. One had a molecular weight of about 70 kDa (band A) while the other two bands migrated at 55 kDa (bands B and C) (Figure 4C). Mass spectrometry showed that band A was mainly composed of fibrinogen α-chain, band B of fibrinogen β-chain, and band C of a fragment of fibrinogen γ-chain (*Online Supplementary Tables S1* and S2, *Online Supplementary Figure S5*).

Perturbed cytoskeletal reorganization causes platelet dysfunction

To induce actin polymerization in control platelets we used jasplakinolide, a natural cyclic peptide that induces actin polymerization and stabilizes actin filaments.[31] We first determined that jasplakinolide is able to induce actin polymerization in control platelets (*Online Supplementary Figure S6*).

Incubation with jasplakinolide did not affect $\alpha_{IIb}\beta_3$ surface expression (Figure 5A), but significantly impaired its activation, induced by ADP (Figure 5B).

Pre-incubation with jasplakinolide impaired, dose-dependently, ADP-induced platelet aggregation (Figure 5C), clot retraction (Figure 5D) and spreading on fibrinogen (Figure 5E). Platelets treated with jasplakinolide (1 μM) resembled patients' platelets with regards to their spreading morphology[11] (Figure 5F). These effects were not a consequence of cell death because platelets were still able to expose P-selectin on their surface upon stimulation with 10 μM ADP (resting platelets 2.92±1.33%; ADP-stimulated platelets 55.03±3.1%; jasplakinolide-treated resting platelets 4.52±1.8%; jasplakinolide-treated ADP-stimulated platelets 57.21±7.9%).

Constitutively activated $\alpha_{IIb}\beta_3$ impairs proplatelet formation

The proportion of mutant-β_3-transduced megakaryocytes extending proplatelets in suspension was not different from that of wild-type megakaryocytes (wild-type megakaryocytes=73.8±19.3%, mutant megakaryocytes=61.4±20.7%; n=5, *P*=ns). However, proplatelet number was reduced and tip diameter was larger in mutant megakaryocytes (Figure 6A,B). Moreover, barbell-proplatelets were significantly more asymmetrical (difference between tips: wild-type megakaryocytes=1±0.9 μm, mutant megakaryocytes=2.5±1.1 μm; n=5, *P*<0.01). Similarly, barbell-proplatelets circulating in peripheral blood of our patient were more asymmetrical than in normal controls (difference between tips: controls=0.5±0.4 μm; patient=1.2±0.8 μm, n=5, *P*<0.05) (Figure 6C).

Mutant β_3-transduced megakaryocytes adhered normally to fibrinogen (wild-type megakaryocytes=51.2±15.3%, mutant megakaryocytes=44.6±20.1%; n=5, *P*=ns) but displayed abnormal spreading, reminiscent of what was previously observed with patient's megakaryocytes[3] (Figure 6D). In contrast, when megakaryocytes were plated on von Willebrand factor, spreading was not different from that of controls (wild-type megakaryocytes=47.9±19.3%, mutant megakaryocytes=50.2±22.6%; n=5, *P*=ns), as already observed with patient's megakaryocytes.[3]

Perturbed actin polymerization impairs proplatelet formation

The proportion of jasplakinolide-treated human megakaryocytes extending proplatelets in suspension was not different from that of control megakaryocytes (vehicle=26.3±2.6%; jasplakinolide=26.9±9.3%, n=3, *P*=ns); however, proplatelet formation was abnormal, with most megakaryocytes displaying a reduced number of proplatelet tips (vehicle=13.9±6.1 tips; jasplakinolide=1.4±1.4 tips, n=3, *P*<0.01) and a reduced tip diameter (vehicle=2.9±0.9 μm; jasplakinolide=1.7±0.8 μm, n=3, *P*<0.01) (Figure 7). Adhesion to fibrinogen of jasplakinolide-treated megakaryocytes was strongly impaired (vehicle=41.4±5.7%; jasplakinolide=4.6±0.5%, n=3, *P*<0.01) and the few adhering megakaryocytes did not spread (Figure 7).

Structural consequences of del647-686

The β-TD connects the lower β-leg of integrin β_3 to the transmembrane portion and consists of an amphipathic α-

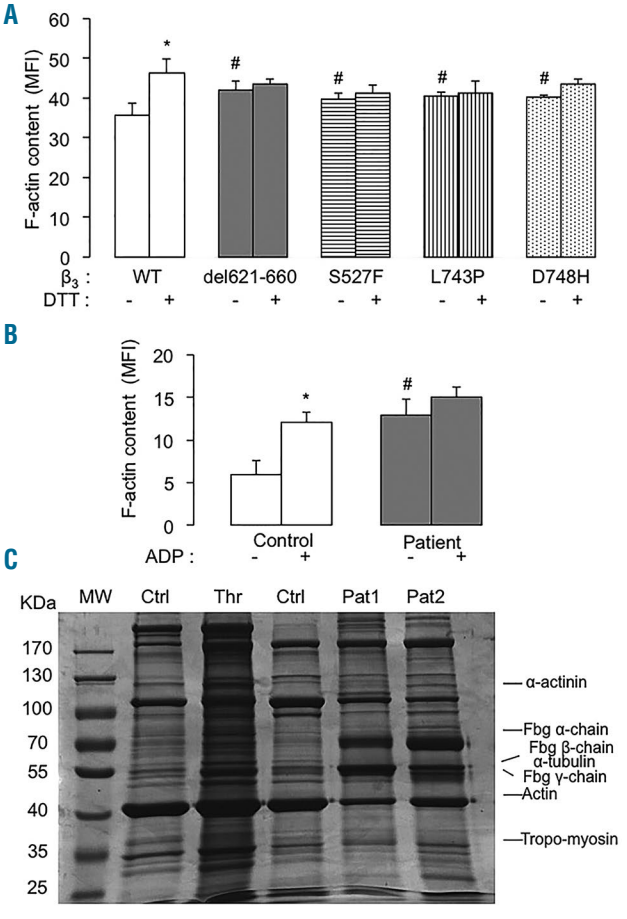

Figure 4. Cytoskeleton dynamics. (A) Polymerized actin (F-actin) content of controls and patients' platelets measured by flow cytometry before and after stimulation with ADP (20 μM) (n=4, **P*<0.05 *vs.* resting, #*P*<0.05 *vs.* control). (B) Polymerized actin (F-actin) content of wild-type (WT) and of different mutant β_3-expressing CHO cells measured by flow cytometry before and after stimulation with DTT (10 μM) (n=6, **P*<0.05 *vs.* resting, #*P*<0.05 *vs.* control). (C) Representative electrophoresis of cytoskeletal proteins from resting control platelets (Ctrl1, Ctrl2), control platelets stimulated with thrombin (thr) and platelets from the two different del647-686 β_3 patients (Pat1, Pat2). Relative quantification (arbitrary units), Control=1; thr=2±0.8, n=3, *P*<0.01; Patients=1.2±0.1 (n=3, *P*<0.01 *vs.* ctrl). Molecular weight marker on the left, identity of the main bands from mass spectrometry analysis on the right. All the proteins found in each band are listed in *Online Supplementary Table S1*.

helix lying across a single β-sheet with four β-strands and four disulfide bonds; it non-covalently associates with the calf-2 domain of α_{IIb} (Figure 8). Residues spanning from Lys532 through Gly690 in the lower β-leg stabilize the interactions with integrins α_v and α_{IIb} in the bent conformation, and mutations of these residues activate $\alpha_{IIb}\beta_3$ and trigger fibrinogen binding.[36] Del647-686 removes the 2, 3, and 4 β-strands, which represent a large interaction interface with the calf-2 domain in both $\alpha_{IIb}\beta_3$[12] and $\alpha_v\beta_3$,[34] thus destroying the β-TD structure (Figure 8), hindering the adoption of the bent conformation. The observation that, despite a 40-amino acid deletion, $\alpha_{IIb}\beta_3$ is still synthesized and expressed on the surface, confirms that β-TD is a domain important for integrin function/activation but not for β_3 synthesis or dimerization with α_{IIb}.[12]

Del647-686 also removes Cys-655 and 663, partners of Cys-608 and 687 in the formation of disulfide bonds. Whether the remaining cysteines would then disulfide-link to one another, exchange with the two α1-helix-loop disulfides, or remain as free sulfhydryls is unknown. However, previous site-directed mutagenesis of Cys-655 and 663 leading to the loss of disulfide bonds, resulted in constitutive activation of $\alpha_{IIb}\beta_3$,[13-15] suggesting that the same mechanism is responsible for the constitutive $\alpha_{IIb}\beta_3$ activation associated with del647-686.

Discussion

Our results show that constitutive activation of $\alpha_{IIb}\beta_3$ due to gain-of-function mutations of *ITGB3*, and the consequent permanent triggering of $\alpha_{IIb}\beta_3$-mediated outside-in signaling, induce a perturbation of cytoskeletal remodeling that leads to platelet dysfunction and impaired proplatelet formation. Starting from the study of platelets from two patients carrying the heterozygous *ITGB3* del647-686 mutation[11] we extended our observations to three additional *ITGB3* gain-of-function mutations[17-19] in order to describe a general mechanism that leads to bleeding in patients with dominant variants of GT.

We show that constitutive activation of $\alpha_{IIb}\beta_3$ leads to permanent triggering of $\alpha_{IIb}\beta_3$-mediated outside-in signal-

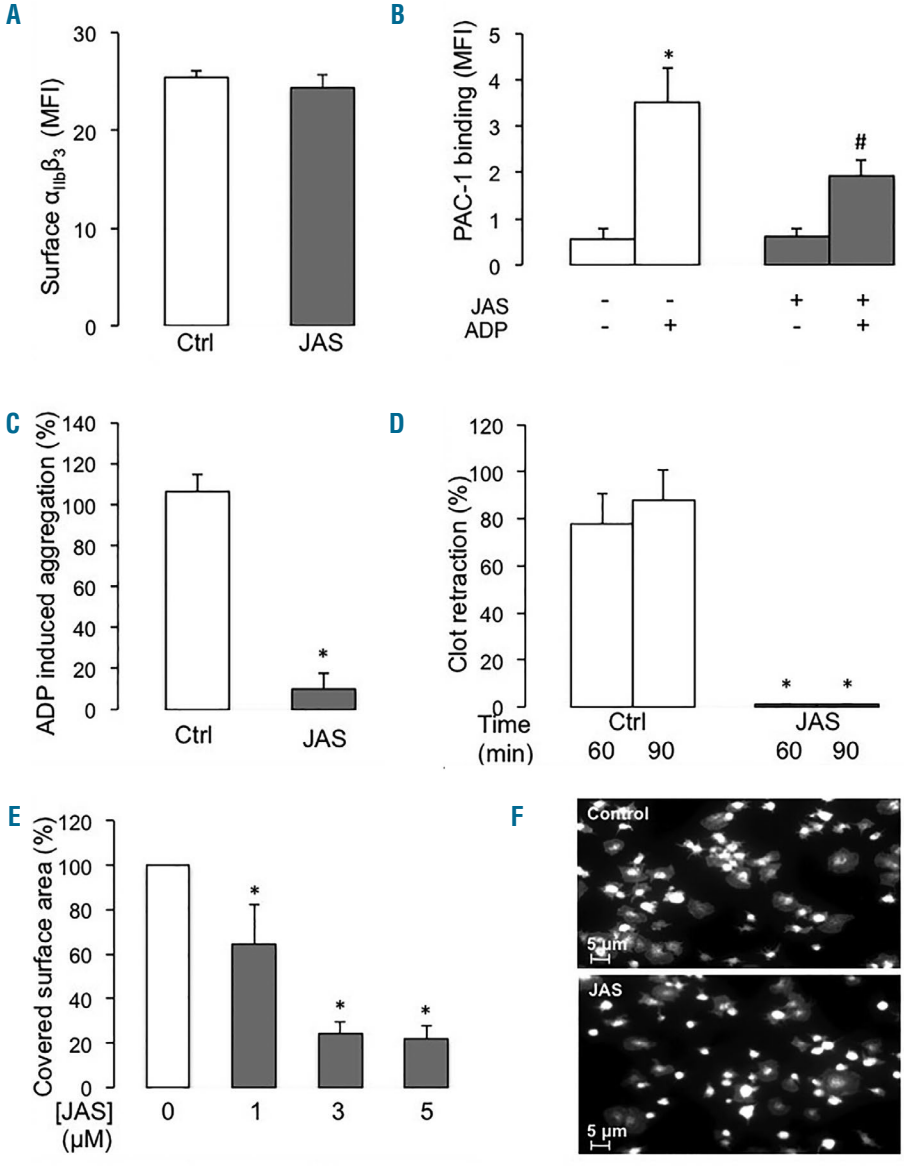

Figure 5. Effect of jasplakinolide on platelet function. (A) $\alpha_{IIb}\beta_3$ expression in platelets treated with jasplakinolide 5 μM (JAS) or its vehicle (CTRL) as assessed by flow-cytometry (n=4, *P*=ns). Data show mean fluorescence intensity. (B) PAC-1 binding to platelets induced by ADP (10 μM) after preincubation with jasplakinolide 5 μM (JAS) or its vehicle (CTRL) (n=4, **P*<0.05 *vs.* resting, #*P*<0.05 *vs.* ctrl ADP). Data show mean fluorescence intensity. (C) ADP-induced aggregation of platelets treated with jasplakinolide 5 μM (JAS) or its vehicle (CTRL) (n=4, **P*<0.01 *vs.* ctrl). (D) Clot retraction mediated by platelets treated with jasplakinolide 5 μM (JAS) or its vehicle (CTRL) (n=4, **P*<0.01 *vs.* ctrl). (E) Platelet spreading on fibrinogen after treatment with 1 μM jasplakinolide. Platelets were stained with the CD41 clone P2 monoclonal antibody. (F) Spreading on fibrinogen of platelets treated with jasplakinolide (JAS) or its vehicle (CTRL) after 60 min of deposition (n=4, **P*<0.01 *vs.* control).

ing, as shown by the phosphorylation of β_3 Tyr773, Tyr785 and focal adhesion kinase in patients' resting platelets and in CHO cells expressing del647-686 β_3, by faster spreading on fibrinogen and spontaneous spreading on von Willebrand factor of patients' platelets, contrarily to control platelets that require $\alpha_{IIb}\beta_3$ activation for spreading on von Willebrand factor.[18,24]

Activation of $\alpha_{IIb}\beta_3$ is physiologically followed by receptor internalization, a way for limiting platelet aggregation.[37,38] We show here that enhanced $\alpha_{IIb}\beta_3$ internalization is the common mechanism leading to reduced surface expression of $\alpha_{IIb}\beta_3$ in patients with GT-like syndromes associated with constitutive activation of $\alpha_{IIb}\beta_3$.[10] In fact, we observed enhanced $\alpha_{IIb}\beta_3$ internalization in CHO cells expressing several different *ITGB3* gain-of-function mutations[17-19] as well as in platelets of del647-686 β_3 patients.

We have previously shown, by flow cytometry using a set of clones directed toward different epitopes of $\alpha_{IIb}\beta_3$, that our patients' platelets express on average 40% residual $\alpha_{IIb}\beta_3$ on their surface if compared with control platelets.[11] We show here that in patients' platelets the ratio between normal and mutant β_3 is maintained on the platelet surface (Figure 1A). The same is observed in CHO cells transfected with both wild-type and mutant β_3 (heterozygous cell model) which express an approximately equal amount of $\alpha_{IIb}\beta_3$ to that of CHO cells transfected with only mutant β_3 (homozygous cell model) (Figure 1A,B). It can, therefore, be speculated that normal $\alpha_{IIb}\beta_3$ is passively internalized along with the mutant one.

Confocal microscopy revealed that in patients' platelets

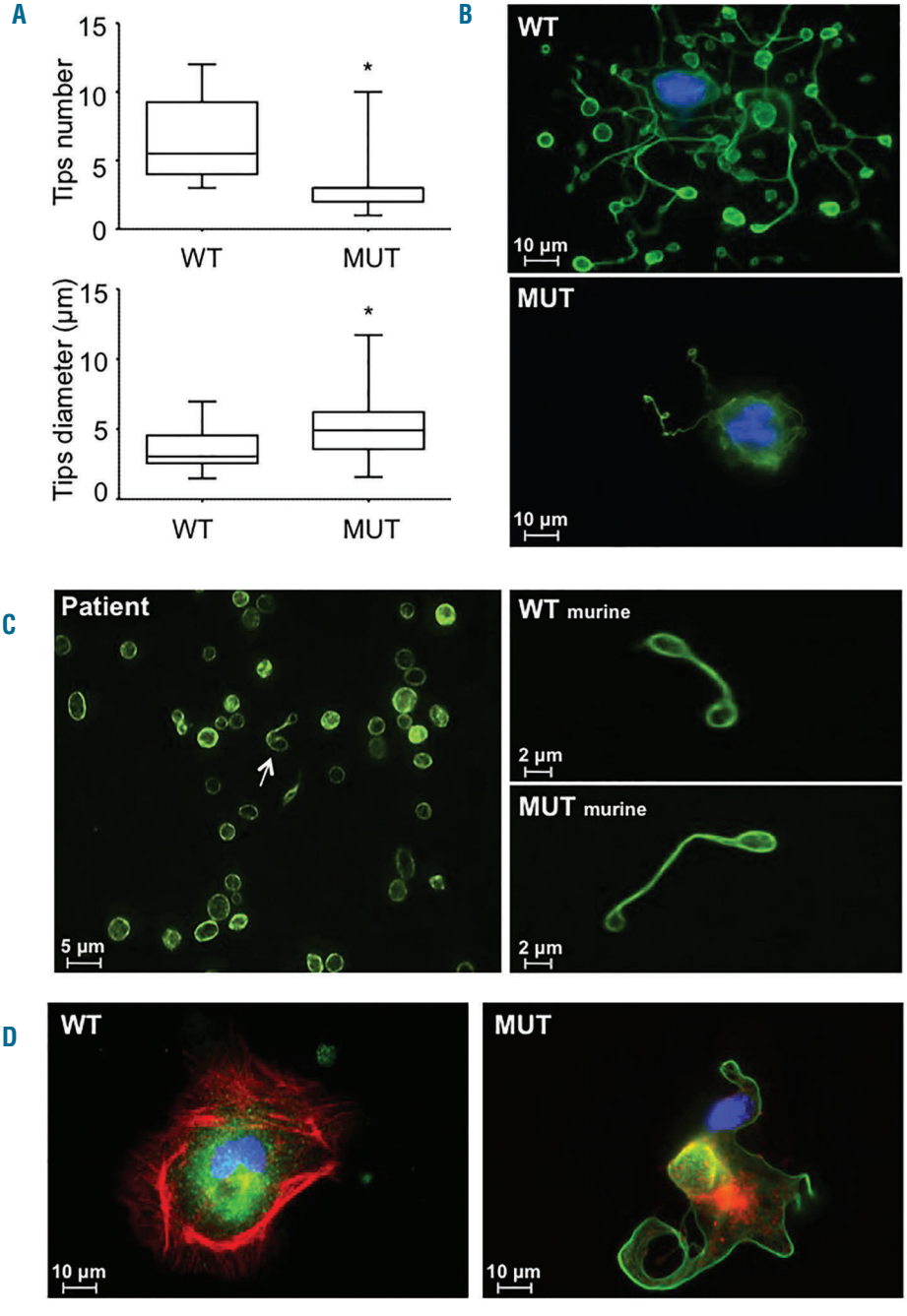

Figure 6. Megakaryocyte spreading and proplatelet formation. (A) Number and diameter of proplatelet tips generated by murine megakaryocytes transduced with the wild-type (WT) or the mutant (MUT) integrin β_3 (*P<0.01 *vs.* WT). Only cells showing both green and red fluorescence (i.e. expressing α_{IIb} and β_3) were analyzed. (B) Representative image of proplatelet formation by wild type- or mutant β_3–expressing murine megakaryocytes. The number of megakaryocytes expressing normal or mutant $\alpha_{IIb}\beta_3$ was comparable (GFP expression in WT 20.2±1.7% *vs.* mutant 19.6±1.3%; Ds-red expression in WT 18.6±1.2% *vs.* mutant 17.7±1.9%; n=5, *P*=ns). Megakaryocytes were stained with a rabbit anti-mouse β_1-tubulin antibody; nuclei were stained with Hoechst. (C) An asymmetric barbell-proplatelet circulating in a patient's peripheral blood is indicated by a white arrow (left). Symmetric barbell-proplatelet generated by wild-type β_3-expressing murine megakaryocytes compared with an asymmetric barbell-proplatelet generated by mutant β_3-expressing murine megakaryocytes (right). Barbell-proplatelets were stained with a rabbit anti-human β_1-tubulin antibody. (D) Representative picture of wild type- or mutant β_3–expressing murine megakaryocytes spreading on fibrinogen. β_1-tubulin was stained with a rabbit anti-human β_1-tubulin, actin was stained by rhodamine-phalloidin, nuclei were stained with Hoechst.

the internal pool of integrin β_3 localizes in α-granules and in other cytoplasmic compartments, probably corresponding to the open canalicular and dense tubular systems,[35] similar to control platelets. Moreover, we show that upon platelet activation, mutant $\alpha_{IIb}\beta_3$ externalizes as well as normal $\alpha_{IIb}\beta_3$, and is, therefore, presumably correctly recycled after internalization.

Enhanced $\alpha_{IIb}\beta_3$ internalization was associated with an increased platelet content of fibrinogen. The intra-platelet fibrinogen pool is largely generated by its internalization mediated by activated $\alpha_{IIb}\beta_3$,[39,40] although internalization by inactive $\alpha_{IIb}\beta_3$ has also been reported.[41] Increased fibrinogen content in the patients' platelets is, therefore, in accordance with constitutively activated $\alpha_{IIb}\beta_3$.

Given that integrin β_3 is a component of the vitronectin receptor $\alpha_v\beta_3$, we measured $\alpha_v\beta_3$ expression on patients' platelets and found it to be comparable to that on control platelets. Therefore, our mutation, like others previously reported,[9] influences $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ expression differently.

In normal conditions, $\alpha_{IIb}\beta_3$-mediated outside-in signaling leads to a reorganization of the cytoskeleton that is required for platelet aggregation, clot retraction and spreading. Cytoskeletal actin reorganization is a finely regulated process with consecutive phases including actin polymerization, which enhances the cytoskeletal rigidity required for platelet shape change, and then depolymerization, which restores the cytoskeletal plasticity required for aggregation and clot retraction.[42] An alteration of actin dynamics and cytoskeletal remodeling can, therefore, impair platelet function.[7,8]

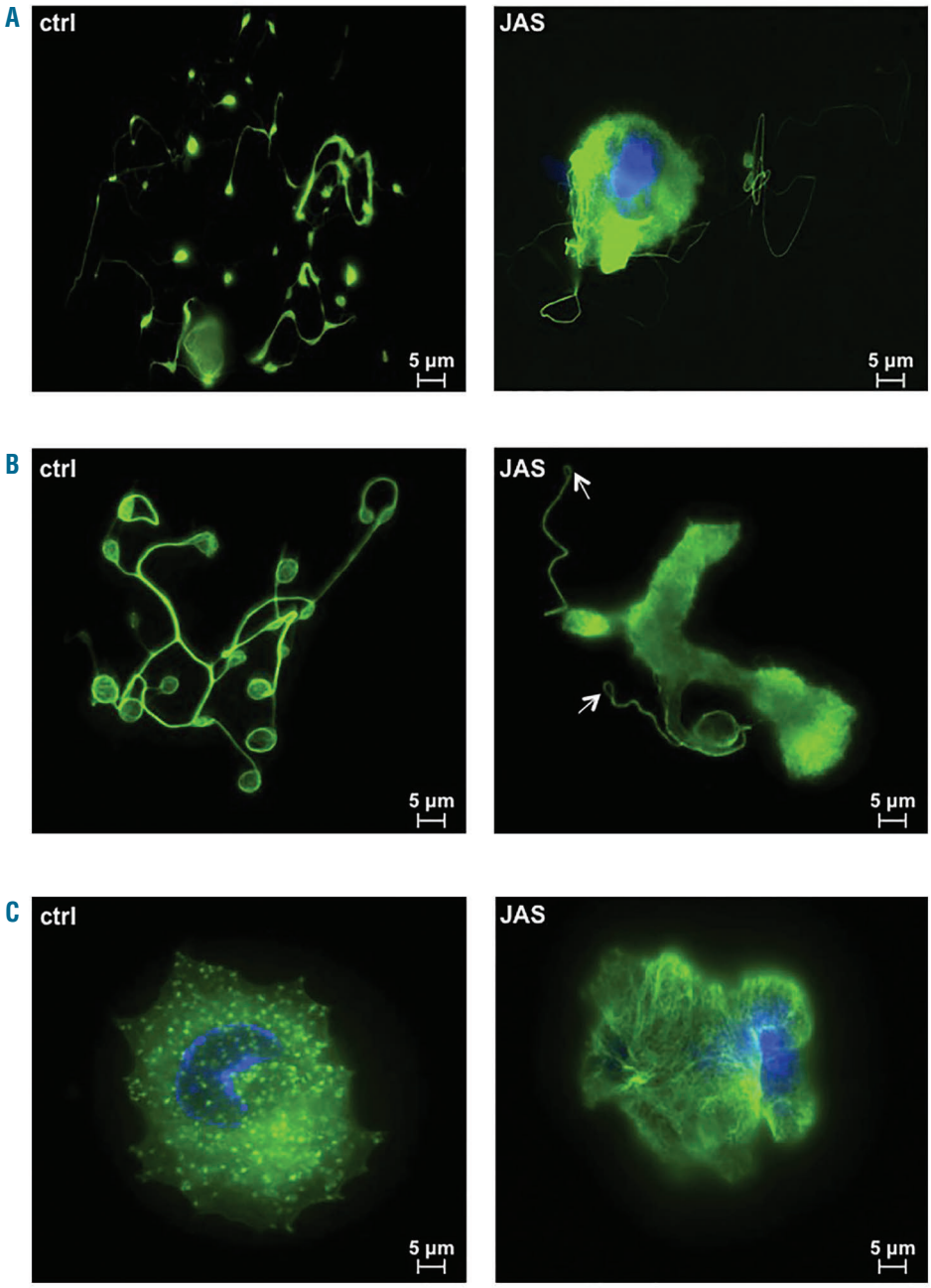

Figure 7. Effect of jasplakinolide on megakaryocyte spreading and proplatelet formation. Representative pictures of proplatelet formation (top and center) and spreading on fibrinogen (bottom) by megakaryocytes treated with vehicle (Ctrl) or 1 µM jasplakinolide (JAS). Megakaryocytes treated with jasplakinolide display a reduced number of proplatelet tips of altered diameter (arrows) and impaired spreading on fibrinogen.

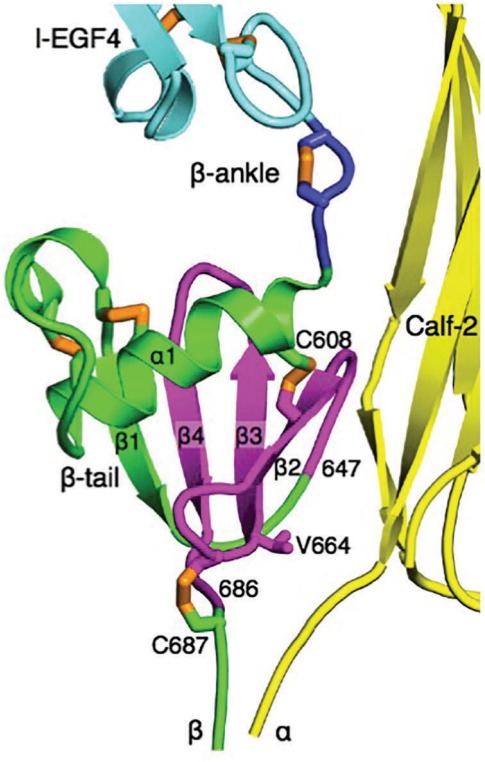

Figure 8. Structural consequences of del647-686. Cartoon diagram showing the structure of the β-tail domain, with each domain in a different color. The deleted region in del647-686 is colored magenta and the remainder of the β-tail domain is colored green. Disulfides and Val-664 side chain are shown as sticks, with sulfur atoms in gold. Ectodomain subunit termini are marked with α and β. The β-tail domain secondary structure elements are variable in length in different β_3 integrin ectodomain structures, and are long in the $\alpha_v\beta_3$ structure depicted here (PDB ID code 4G1E).[36]

We show here that CHO cells expressing *ITGB3* gain-of-function mutations, as well as del647-686 β_3-bearing platelets, have an increased F-actin content under resting conditions and show impaired clot retraction, suggesting that actin turnover is arrested at the stage of polymerization due to permanently triggered outside-in signaling. This is in line with the earlier spreading on fibrinogen of mutant platelets becoming defective at later time points, because actin turnover is arrested at the stage of polymerization and further cytoskeletal remodeling is no longer possible. Moreover, patients' platelets had an enhanced cytoskeletal-associated protein content as compared with control platelets, and fibrinogen α, β and γ chains were found to be associated with the cytoskeleton.

In order to assess whether impaired cytoskeletal remodeling recapitulates the platelet features associated with $\alpha_{IIb}\beta_3$-activating *ITGB3* mutations we used jasplakinolide, a natural cyclic peptide that induces actin polymerization by promoting actin nucleation and by preventing depolymerization of stress fibers.[31] Jasplakinolide-treated control platelets showed impaired $\alpha_{IIb}\beta_3$ activation, reduced aggregation, altered spreading on fibrinogen and clot retraction, a phenotype resembling that of del647-686 platelets.[11] Altogether, these data are compatible with a model in which $\alpha_{IIb}\beta_3$ constitutive activation causes the arrest of cytoskeletal remodeling at the stage of polymerization, thus impairing platelet aggregation, clot retraction and spreading. On the other hand, reduced $\alpha_{IIb}\beta_3$ surface expression does not seem to contribute significantly to platelet dysfunction because incubation of normal platelets with jasplakinolide induced the same platelet dysfunction observed in patients with variant GT without affecting $\alpha_{IIb}\beta_3$ surface expression. Moreover, $\alpha_{IIb}\beta_3$ internalization is not triggered by actin polymerization but depends on the constitutive activation of the receptor.

Cytoskeletal remodeling is also crucial for proplatelet formation: in fact actin polymerization leads to the amplification of proplatelet ends, thus increasing tip number,[43] and regulates platelet size.[35,36] Impaired actin remodeling in megakaryocytes may thus perturb platelet formation, and in fact defects in actin-related proteins were shown to lead to thrombocytopenia and to the production of platelets with altered dimensions.[44-47] We induced actin polymerization by incubating control megakaryocytes with jasplakinolide and indeed we observed impaired proplatelet formation.

Murine megakaryocytes transduced with del647-686 human β_3 and wild-type human α_{IIb} showed impaired proplatelet formation, with a reduced number of abnormally large proplatelet tips. These data confirm our previous observations with peripheral blood $CD34^+$-derived patients' megakaryocytes[3] and prove that the 2134+1 G>C mutation is the cause of macrothrombocytopenia. We also observed that barbell-proplatelets produced by transduced murine megakaryocytes are asymmetrical, similar to those found in patients' peripheral blood. This is therefore probably responsible for the platelet anisocytosis observed in patients with the del647-686 mutation.[11]

Our observations confirm that $\alpha_{IIb}\beta_3$ plays a role in proplatelet formation[3,18,48] and show that when outside-in signaling is constitutively triggered, cytoskeletal reorganization is disturbed and altered proplatelet formation and preplatelet maturation occur.

In conclusion, we show that gain-of-function mutations of *ITGB3* generating constitutive activation of $\alpha_{IIb}\beta_3$ lead to receptor internalization and to the arrest of cytoskeletal remodeling in platelets and megakaryocytes which, in turn, generate platelet dysfunction and perturb the finely regulated process of proplatelet formation leading to macrothrombocytopenia. Reduced platelet number and platelet dysfunction in patients with dominant variants of GT are both consequent to the cytoskeletal perturbation induced by the constitutive $\alpha_{IIb}\beta_3$-mediated outside-in signaling.

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